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Title: DNA Damage-Inducible Gene Expression and Formation of 5-Fluorouracil-Resistant Mutants in *Escherichia coli* Exposed to 2-Dodecylcyclobutanone

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DNA Damage–Inducible Gene Expression and Formation of 5-Fluorouracil–Resistant Mutants in *Escherichia coli* Exposed to 2-Dodecylcyclobutanone

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ABSTRACT: 2-Dodecylcyclobutanone (2-DCB) is formed by the radiolysis of palmitic acid and is present at low part-per-million (approximately 0.1 μg/g) levels in irradiated meat products. Recently, equivocal results obtained using a DNA strand breakage test, the Comet Assay, raised the possibility that 2-DCB could be a weak genotoxin. To more accurately assess 2-DCB's potential genotoxicity, it was tested for the ability to increase expression of the DNA damage–inducible genes *dinD*, *nfo*, *recA*, and *umuDC* using *Escherichia coli* that contained promoter/β-galactosidase reporter constructs, and for the ability to increase the formation of 5-fluorouracil (5-FU)–resistant mutants. When *E. coli* was exposed to 125, 250, 500, and 1000 μg/mL 2-DCB, with and without exogenous metabolic activation, no increase in *dinD*, *nfo*, *recA*, or *umuDC* gene expression, as measured by an increase in β-galactosidase activity, was observed. In addition, 2-DCB did not increase the formation of 5-FU–resistant mutants in *E. coli*, with and without exogenous metabolic activation, at the same concentrations. No evidence of 2-DCB–associated genotoxic activity was detected in this study.

Keywords: 2-dodecylcyclobutanone (2-DCB), irradiation, mutation, DNA, gene expression

Introduction

Exposure of foods containing fatty acids, such as meat and poultry, to ionizing radiation leads to the formation of compounds called 2-alkylcyclobutanones (2-ACBs), which are not detectable in nonirradiated meat products (Boyd and others 1991). The most abundant of the 2-ACBs in meat is 2-dodecylcyclobutanone (2-DCB) (Crone and others 1992). Cleavage of the acyl-oxygen bond of palmitic acid by ionizing radiation leads to its cyclization, resulting in a molecule with the same number of carbon atoms as palmitic acid with an alkyl group in the s ring position, or 2-DCB ($C_{16}H_{30}O$; FW 238.41) (Figure 1). A person consuming 125 g of irradiated ground beef would be expected to consume approximately 6.0 μg of 2-DCB, or 0.00006 mg/kg for a 100-kg adult, or 0.00024 mg/kg for a 25-kg child (calculated from Public Citizen 2003).

Recently, it was claimed that 2-DCB was a potential genotoxin (Delincee and Pool-Zobel 1998; Delincee and others 1999). When rats were fed 2-DCB (14.9 mg/kg), a weak genotoxic response (DNA strand breakage using the Comet Assay) was obtained in rat colon cells (Delincee and others 1999). In the same study, a 2-DCB dose of 1.12 mg/kg did not increase DNA strand breakage in rat colon cells. Similar results were obtained when human and rat colon cells were exposed in vitro to 2-DCB (>1 mg/mL) (Delincee and Pool-Zobel 1998). However, the Comet Assay sometimes yields false-positive results due to chromosome fragmentation when cytotoxicity (cell death) is induced (Tice and others 2000; Health Canada 2003). When 2-DCB was retested at noncytotoxic concentrations, no increase in DNA strand breakage was observed in human colon cell lines (Burnouf and others 2002). However, in the same study, 2-DCB induced DNA damage in HELA cells when significant cell killing occurred. Because of these and other concerns, the authors cautioned against interpretation of their results to mean that either 2-DCB or irradiated meat is carcinogenic (Burnouf and others 2002; Delincee 2003). Claims of 2-DCB's alleged genotoxicity have taken on added recent importance following the introduction, on a voluntary basis, of irradiated ground beef into the USDA's Natl. School Lunch Program in 2004. Some groups opposed to food irradiation have claimed, because of the results obtained using the Comet Assay, that 2-DCB is mutagenic and that irradiated meats therefore promote cancer (ACO 2001; Public Citizen 2002, 2003; Au 2002).

To more accurately assess the mutagenic and recombinogenic potential of 2-DCB, the compound has been tested using multiple short-term genotoxicity assays. In previous work, 2-DCB did not induce mutations in 2 bacterial reverse mutation assays, the *Salmonella* mutagenicity test and the *Escherichia coli* Tryptophan (TRP) reversion assay (Sommers 2003; Sommers and Schiestl 2004). When 2-DCB was tested for the ability to cause chromosome rearrangement in the yeast DEL assay, no increase in recombination rate was observed (Sommers and Schiestl 2004). In the yeast DEL assay, recombination is induced by the formation of DNA strand breaks, a substrate for initiation of recombinational repair in yeast (Sommers and others 1995).

Other methodologies used to assess a compound's genotoxicity include analysis of DNA damage–inducible gene expression, which is sometimes able to identify genotoxins missed by the bacterial reverse mutation assays (Quillardet and Hofnung 1985; Orser and others 1995a, 1995b; Vollmer and others 1997; Rosenkranz and others 1999). In this study, the ability of 2-DCB to increase the expression of DNA damage–inducible genes in *E. coli* that contained stress-inducible promoters fused to β-galactosidase reporter genes was examined (Orser and others 1995a, 1995b). In addition, the ability of 2-DCB to induce the formation of 5-fluorouracil (5-FU)–resistant mutants in *E. coli* was also quantified.

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Materials and Methods

Bacterial strains

Escherichia coli strains, SF1 dinD::lacZ tn10::tolC, SF1 recA::lacZ tn10::tolC, SF1 umuDC-lacZ tn10::tolC, and SF1 nfo-lacZ tn10::tolC, were obtained from Xenometrix, Inc. (Boulder, Colo., U.S.A.). The bacteria contain both promoter-β-galactosidase fusions and transposon (Tn10) disruptions of the *tolC* gene, which increases the permeability of the *E. coli* cell wall to test compounds (Orser and others 1995a). The *E. coli* strains were propagated on brain heart infusion (BHI) agar (Difco, Inc., Sparks, Md., U.S.A.) at 37°C and maintained at 0°C to 4°C until ready for use.

Chemicals

2-Dodecylcyclobutanone (2-DCB) was obtained from the Fluka subsidiary of Sigma-Aldrich, Inc. (St. Louis, Mo., U.S.A.). Methyl methanesulfonate (MMS), dimethylsulfoxide (DMSO), polymyxin B sulfate, and 5-FU were obtained from Sigma-Aldrich, Inc. 2-Aminoanthracene (2-AA) and 4-nitroquinolone-N-oxide (4-NQNO) were obtained from Moltox, Inc (Boone, N.C., U.S.A.).

Exogenous metabolic activation

To simulate mammalian physiology and metabolism, exogenous metabolic activation was used for both the gene expression and mutagenesis assays (Ames and others 1975). Aroclor 1254–induced S9 fraction, from male Sprague-Dawley rats, was obtained from Moltox, Inc., as were NADPH regeneration systems A and B. S9 fraction 20% (v/v) was prepared fresh before each experiment according to the manufacturer's instructions.

Bacterial cultures

Single colonies of each tester strain were inoculated into 5 mL BHI medium (Difco, Inc.) in sterile borosilicate glass test tubes and incubated approximately 16 h, 37°C, with150 rpm in a shaking incubator.

Microplate set-up

Standard 8 row by 12 column 96-well microtiter plates (Nunc) were used for the experiments (Orser and others 1995a, 1995b). Column 1 was filled with 200 µL/well of BHI medium for use as a blank control for the microplate reader. Column 2 was for the untreated, no-chemical control. Columns 3 through 10 were used for exposure to test compound. Column 11 was untreated to avoid cross-contamination with positive controls, and column 12 was used for positive control compounds. Each row (A-D) contained an *E. coli* strain with a different stress-inducible reporter construct.

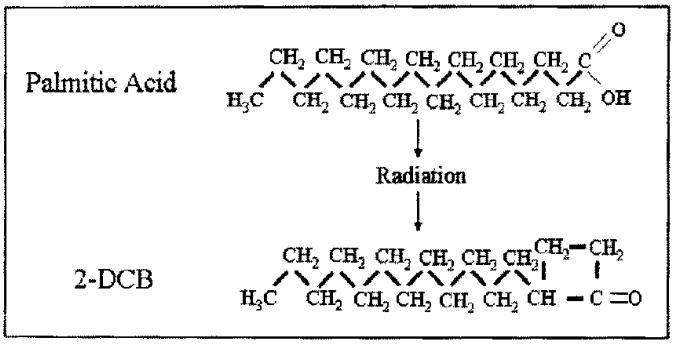

Figure 1—Structure of palmitic acid and 2-dodecylcyclobutanone (2-DCB) as determined by LeTellier and Newar (1972)

Determination of gene expression

For experiments without exogenous metabolic activation 170 µL of BHI medium was added to Columns 2 through 12. For experiments with metabolic activation, 150 µL of BHI medium was used. Then 10 µL (10^6 cells) of bacterial culture was added to each of those wells. The microplates were then placed in a 37°C shaking incubator, 150 rpm, for 1 h to allow the bacteria to begin logarithmic growth (Orser and others 1995a, 1995b). After the 1-h incubation period, 20 µL of solvent (25% DMSO in water) was added to column 2. 2-DCB, prepared as 10X stocks in 25% DMSO, pre-diluted in a separate "chemical plate" was then added to columns 3 through 10 to achieve the desired 2-DCB concentrations (Orser and others 1995a, 1995b). Twenty microliters of positive control compound, resuspended in 25% DMSO, was then added to column 12. Twenty microliters of 20% S9 fraction was then added to those microplate wells, for a final concentration of 2%, for those experiments that required exogenous metabolic activation. Columns 2 through 10 and 12 contained final volumes of 200 µL.

After addition of chemicals and S9 fraction, the optical density (600 nm) was recorded using a BioRad model 2550-E1A microplate reader. The bacteria were then exposed to the chemical for 1.5 h, 37°C, 150 rpm, in a shaking incubator to allow for RNA production and translation of the β-galactosidase protein (Orser and others 1995a, 1995b). After the 1.5-h incubation period, a 2nd optical density measurement (600 nm) was taken. Fifty microliters of a 400 mg/mL polymyxin B sulfate, suspended in sterile water, was added to each well and allowed to sit for 15 min, to permeablize the bacterial cells. After the permeablization step, 50 µL of cells from each well were added to a microplate that contained 200 µL Z-buffer containing 1 g/L ONPG (Orser and others 1995a, 1995b). Column 1 contained 250 µL for Z buffer and was used as the blank for the microplate reader. An optical density reading (420 nm) was then recorded, the enzymatic reaction allowed to continue for 0.5 h, and then a 2nd optical density measurement (420 nm) was recorded. Enzymatic activity was determined as previously described (Orser and others 1995a, 1995b). Two duplicate plates were used per experiment, with the experiment was completed independently 3 times.

Mutation frequency

E. coli strain SF1 umuDC::lacZ Tn10::tolC was used for the determination of mutation frequency. Bacterial strain, test compound, S9 fraction, and positive control compound were each prepared as described previously. Exposure to the test compound was also performed in microtiter plates as described previously, but the time was extended to 4 h to allow for DNA replication and fixation of mutations within the genome. To assess culture viability after exposure to chemicals, the cells were serially diluted in Butterfield's phosphate buffer and 50 µL of the samples plated on Vogel-Bonner minimal agar medium containing 5 µg/mL 5-FU for selection of nucleoside analog resistant mutants (Skopek and Thilly 1983) and on Vogel-Bonner minimal medium supplemented with 0.5% casamino acids (Difco, Inc.) to assess culture viability. The plates were then incubated for 3 d at 37°C, and the number of colony-forming units (CFU)/plate were scored. The mutation frequency is defined as the fraction of 5-FU revertants per number of viable cells. Each experiment (2 duplicate samples per test compound concentration) was conducted independently 3 times.

Statistical analysis

Duplicate samples were used for each experiment. Each experiment was conducted independently 3 times. The descriptive statistics function of Microsoft Excel (Microsoft, Inc., Redmond, Wash., U.S.A.) was used to perform analysis of variance (ANOVA) and Student *t* test.

Table 1—Induction of DNA damage–inducible genes by 2-dodecylcyclobutanone[a]

		2-Dodecylclobutanone μg/mL					
Reporter	S9	0	125	250	500	1000	Pos. control
dinD-lacZ	–	4.37 (±1.43)	3.48 (±0.84)	3.50 (±1.28)	3.36 (±2.17)	1.21 (±0.67)	28.8 (±10.1)
	+	5.11 (±0.30)	2.08 (±0.79)	1.74 (±0.65)	1.69 (±0.62)	4.80 (±2.03)	54.4 (±17.5)
recA-lacZ	–	17.4 (±6.12)	13.9 (±5.54)	12.3 (±6.08)	13.5 (±5.61)	13.8 (±8.68)	57.3 (±19.3)
	+	7.55 (±1.9)	5.27 (±1.21)	4.50 (±1.67)	5.23 (±1.24)	4.51 (±1.15)	65.1 (±5.67)
nfo-lacZ	–	3.95 (±1.77)	3.58 (±0.85)	2.83 (±0.44)	1.84 (±0.40)	21.0 (±9.01)	87.7 (±20.5)
	+	21.8 (±2.40)	13.6 (±3.00)	13.5 (±3.03)	13.2 (±2.27)	14.8 (±1.33)	104 (±10.9)
umuDC-lacZ	–	9.89 (±2.91)	10.6 (±3.07)	9.86 (±3.77)	10.4 (±4.13)	10.6 (±5.25)	57.1 (±11.2)
	+	3.87 (±1.65)	2.94 (±1.64)	3.10 (±1.42)	1.73 (±0.82)	2.10 (±1.57)	48.0 (±5.42)

[a]The results, shown as β-galactosidase activity, were tabulated from 3 independent experiments. Positive controls for *dinD*, *recA*, and *umuDC*, reporter constructs without S9 fraction were130 μg/mL methyl methanesulfonate (MMS) and 5 μg/mL 4-nitroquinolone-N-oxide (4-NQNO) for *nfo*. The positive control for all strains with S9 fraction was 10 μg/mL 2-aminoanthracene (2-AA).

Table 2—Induction of 5-fluorouracil–resistant mutants in *Escherichia coli* (SF1 umuDC::lacZ Tn10::tolC) exposed to 2-dodecylcyclobutanone, with and without exogenous metabolic activation[a]

	Frequency (× 10^{-6}) of 5-fluorouracil–resistant mutants (±SEM)					
2-DCB (μg/mL)	0	125	250	500	1000	Pos. control
No S9 fraction	0.74 (±0.17)	1.10 (±0.33)	0.96 (±0.87)	0.83 (±0.16)	0.79 (±0.17)	12.6 (±2.88)
2% S9 fraction	1.09 (±0.14)	0.95 (±0.14)	0.88 (±0.16)	0.97 (±0.14)	0.90 (±0.22)	7.43 (±1.99)

[a]Results were tabulated from 3 independent experiments. Positive control compounds were 130 μg/mL methyl methanesulfonate (MMS) without S9 fraction, and 10 μg/mL 2-aminoanthracene (2-AA) with S9 fraction. 2-DCB = 2-dodecylcyclobutanone.

Results and Discussion

Gene expression profiling has been used extensively for the determination of chemical genotoxicity and is capable of detecting many genotoxins that are not detectable using bacterial reverse mutation assays (Quillardet and Hofnung 1985; Orser and others 1995a, 1995b; Rosenkranz and others 1999). The UmuDC proteins of *E. coli* allow DNA polymerases to bypass DNA adducts during DNA replication in an error-prone DNA repair pathway (Quillardet and Hofnung 1985; Smith and others 1990; Ohta and others 1999). The RecA protein is both an ssDNA binding protein and a regulator of SOS-inducible DNA repair pathways in *E. coli* (Kenyon and Walker 1990; Weiseman and others 1994). Nfo (DNA endonuclease IV) is required for the repair of oxidative DNA damage (Ljungquist 1977; Saporito and Cunningham 1988), whereas DinD (OrfA/PyrE/pcsA) protein is involved in DNA replication and resolution of recombination intermediates (Ohmori and others 1995). Transcription of RNA from each of these genes is increased after exposure of *E. coli* to xenobiotics that induce DNA damage (Kenyon and Walker 1990; Orser and others1995a, 1995b). 2-DCB was not able to induce gene expression, as measured by increased β-galactosidase activity levels, at concentrations of 9, 18, 36, 63, 125, 250, 500, and 1000 μg/mL (1 g/kg) in *E. coli* SF1 strains containing the promoter/β-galactosidase reporter constructs (Table 1), with or without exogenous metabolic activation ($n = 3$, $\alpha = 0.05$) as determined by Student *t* test and ANOVA. In contrast, β-galactosidase activity was increased in the *E. coli* cells exposed to their respective positive control compounds (Table 1).

2-DCB has previously tested as negative for the ability to induce mutations in bacterial reverse mutation assays including the *Salmonella* mutagenicity test or the *E. coli* TRP reverse mutation assay (Sommers 2003; Gadgil and Smith 2004; Sommers and Schiestl 2004). Those assays measure the ability of a xenobiotoc to revert specific mutations in genes required for amino acid synthesis from auxotrophy from prototrophy, most often in DNA repair–deficient bacterial strains. In contrast, 5-FU–resistant mutants in *E. coli* or *Salmonella* are formed when a null mutation is fixed within the DNA sequence of the 0.551-kb uracil-phosphoribosyltransferase gene, which would normally convert 5-FU to a toxic metabolite within the bacterium (Skopek and Thilly 1983; Hayashi and others 2001; Koyama and others 2003). The DNA target available for mutagenesis in these forward mutation frequency assays is much larger than that in bacterial reverse mutation tests, an entire gene, and the DNA repair proficiency of the *E. coli* SF1 strain used in this study allows for detection of oxidative DNA-damaging xenobiotics different from that of many DNA repair–deficient bacteria (Levin and others 1984; Berglin and Carlsson 1986). Although 2-DCB reduced cell viability to 27% at the highest concentration (1 mg/mL or 1 g/kg) after the 4-h incubation period, no increase in mutation frequency (formation of 5-FU–resistant colonies) was observed in the *E. coli* SF1 strain exposed to 0.125, 0.25, 0.5, or 1.0 mg 2-DCB for 4 h, with or without exogenous metabolic activation, as determined by Student *t* test ($n = 3$, $a = 0.05$) (Table 2).

Results obtained in this study are consistent with those obtained in other short-term genotoxicity tests in which 2-DCB was unable to induce either mutagenesis or DNA strand break–induced chromosome rearrangements (Sommers 2003; Gadgil and Smith 2004; Sommers and Schiestl 2004). Differences in the results obtained in these tests may be due to differences in mammalian physiology as opposed to those of bacteria or yeast; however, inclusion of exogenous metabolic activation, S9 fraction from Aroclor 1254–induced rats, did not influence the nature of the results that were obtained.

Another more likely possibility was that the equivocal results obtained using the Comet Assay were due to nongenotoxic cell death caused by 2-DCB (Tice and others 2000; Health Canada 2003). That possibility is even more likely when one considers results obtained in long-term, multigeneration feeding studies in animals using irradiated foods. Swallow (1991) reported that animals used for toxicological research, fed diets of radiation-sterilized foods for 40 generations, suffered no ill effects from consumption of irradiated foods. Thayer and others (1987) reported that rodents fed diets of radiation-sterilized chicken meat for 3 generations did not suffer an increased risk of cancer or birth defects. The same study also showed radiation-sterilized chicken meat to be negative in the *Salmonella* mutagenicity test. Eekelen and others (1971, 1972) con-

ducted single- and multiple-generation feeding studies in rats using radiation-pasteurized chicken meat without finding adverse effects due to consumption of the irradiated chicken diet. Poling and others (1955) reported no evidence of changes in survival, histopathology, or reproduction in 3 generations of rats fed radiation-sterilized ground beef.

Conclusions

2-DCB did not increase expression of DNA damage–inducible genes in *E. coli* or the formation of 5-FU–resistant mutants. The results of this study, when considered in combination with previous mutagenicity and recombinagenicity tests completed using elevated concentrations of 2-DCB, and previously conducted long-term multigeneration feeding studies in animals using irradiated meats, call into question the claims of irradiated foods causing cancer in humans.

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